Binding-Dissociation Properties of Potato Tuber Cell Wall-Associated β-*N*-acetylglucosaminidase

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The binding-dissociation properties of an endogenous cell wall protein, β-GlcNAcase, was compared to an artifactually bound basic protein (cytochrome *c*) and acidic proteins (bovine serum albumin, α-lactalbumin, β-lactoglobulin and cytosolic β-GlcNAcase). Salt dissociation curves with monovalent, divalent and trivalent salts all indicated that the endogenous cell wall enzyme binds much tighter to the wall than does any artificially bound protein. At high ionic strength (I=1.5), ammonium sulfate was as efficient as NaCl, KCl and LiCl in dissociating the cell wall enzyme. The pH of the dissociation medium only has an effect on the dissociation of cell wall enzymes when the ionic strength of the buffer is low. The binding of protein to purified cell walls is pH dependent in the physiological range only if the protein has an acidic isoelectric pH.

Key words: Binding and dissociation of proteins — Cell walls — Ionic strength — pH — Potato tubers.

Although cell wall-associated enzymes are frequently solubilized with high molarity salt (Huber and Nevins 1982, Scott and O'Neill 1984), the optimum conditions which affect the binding-dissociation of cell wall enzymes from purified cell walls have not been established. Furthermore, very little information is available on the binding-dissociation properties of cytosolic proteins which potentially can bind to the cell wall during any cell disruption process. In this study, we have used a recently developed cell wall isolation procedure which uses a Parr nitrogen bomb to maximize cellular disruption and minimize any oxidation processes which can lead to browning and protein denaturation (Nagahashi and Seibles 1986). Cell walls purified by this procedure were used to investigate the roles of pH, ionic strength, and cation specificity on the binding-dissociation properties of potato tuber cell wall-associated β-*N*-acetylglucosaminidase (β-GlcNAcase) in comparison to the cytosolic β-GlcNAcase and other artificially bound proteins. Cytosolic β-GlcNAcases are known to be involved in glycoprotein processing and the cell wall associated enzyme may be involved in the host defense system (Nagahashi et al. 1990). This study indicates that the cell wall-associated β-GlcNAcase has properties which clearly distinguish this isozyme from the cytosolic form.

Materials and Methods

Cell wall isolation and purification—Cell walls were isolated from Russet potato tubers using the Parr nitrogen bomb as the major cell disruption step (Nagahashi and Seibles 1986). Tubers were initially washed, peeled, sliced and homogenized in alkaline medium (Nagahashi and Garzarella 1988) in a Waring blender for 60 s at 4°C. Intact tissue clumps and broken cell walls were trapped on cheesecloth and washed three times. After the last washing, the crude particulate fraction was suspended in fresh homogenization medium and placed in a nitrogen bomb. After equilibrating at 1,500 psi for 15 min at 4°C, the contents were extruded from the bomb and washed six times to remove starch bodies and cellular membrane components from the large fragments of walls. These isolated cell walls were judged to be highly purified by electron microscopy and lack of cytoplasmic and membrane marker activity (Nagahashi and Seibles 1986).

Partial purification of the cytosolic β-GlcNAcase—The soluble homogenate from the Waring blender was combined with the soluble homogenate from the nitrogen bomb and centrifuged at 40,000×*g* for 30 min to pellet membranes. The supernatant was subjected to sequential ammonium sulfate steps. Most of the cytosolic β-GlcNA-

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case precipitated between 40 and 80% ammonium sulfate. The pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.0 and dialyzed against deionized-distilled water for 24 h. The sample was applied to a sephacryl G300 column and eluted with 50 mM phosphate buffer, pH 7.0 in 0.1 M NaCl. A single peak was collected, concentrated in an Amicon pressure apparatus with a PM 10 membrane, and isoelectric focussed as described earlier (Nagahashi and Seibles 1986). A single peak was observed with an isoelectric pH at 5.2. The enzyme was purified approximately 30 fold and was used as the partially purified enzyme.

Enzyme assays—Enzyme assays were performed as described previously (Nagahashi et al. 1990). When cell wall enzymes were assayed *in muro*, 0.1 to 0.5 mg dry weight cell wall (0.1 to 0.2 ml of cell wall in water) was used per assay and the cell walls were concentrated or diluted as reported (Nagahashi et al. 1990).

Binding-dissociation assays—One ml aliquots of purified cell walls (0.4 to 0.6 mg dry wt) were placed in test tubes containing various salt solutions with increasing concentration. The cell walls were stirred with a glass rod every ten minutes and after 2 h, the test tubes were centrifuged for 3 min at 1,000 × *g* to pellet the cell walls. Alternatively, a 10 ml or 20 ml suspension of cell walls and salt were stirred in 25 ml beakers on a multipoint magnetic stirrer at room temperature. Salt dissociation curves were usually performed at room temperature for 1 h however, maximum dissociation was achieved in 30 min with the magnetic stirring bar setup. If the dissociation was carried on overnight, the samples were kept at 4°C.

Cell wall-dissociated β-GlcNAcase was determined by measuring the activity released to the supernatant with salt treatment. Cytochrome *c* was artifactually bound to purified cell walls and the salt dissociated cyt *c* was determined by pelleting the cell walls and reducing an aliquot of the supernatant with sodium dithionite immediately before reading at 550 nm. Quantification was done by comparing to a reduced cyt *c* standard (Nagahashi and Garzarella 1988).

The binding of lysozyme, α-lactalbumin, β-lactoglobulin and bovine serum albumin, cytochrome *c*, cell wall β-GlcNAcase, and cytosolic β-GlcNAcase were performed by adding a specific amount of protein (mg for all proteins except enzymes where a known total enzyme activity was added) to a 10 ml aliquot of cell walls. The binding was determined by measuring the amount of enzyme activity or protein left in the supernatant after stirring for 1 h at room temperature. Cytochrome *c* and β-GlcNAcase activity were quantified as described above. The rest of the proteins were quantified by using a modified Lowry procedure for the colorimetric measurement of proteins (Markwell et al. 1978). A standard curve was constructed for each protein used. After the binding period, the amount of protein bound was calculated by subtracting the residual protein in the supernatant from the starting concentration. The difference was assumed to be bound protein. Binding assays were usually performed at room temperature in water. In some cases, the binding assays were performed at various pH in a 25 mM sodium citrate-citric acid buffer system or a KH_2PO_4–K_2HPO_4 buffer system at increasing molarity.

Results

Dissociation of β-GlcNAcase and artificially bound cytochrome c from cell walls with NaCl—Cytochrome *c* was allowed to bind to purified cell walls as described above. This protein was chosen because it was not present in plant cell walls and it had an isoelectric pH of 10.4 which was similar to the cell wall associated β-GlcNAcase (Nagahashi and Seibles 1986). At low concentrations of NaCl, cytochrome *c* was readily dissociated from the cell wall (Fig. 1A). By 0.1 M NaCl, close to 80% of the cyt *c* was dissociated. In contrast, the cell wall associated enzyme, β-GlcNAcase, was not released at this concentration of salt (Fig. 1A). To release 80% of the cell wall enzyme, the NaCl concentration had to be 4 to 5 times higher than

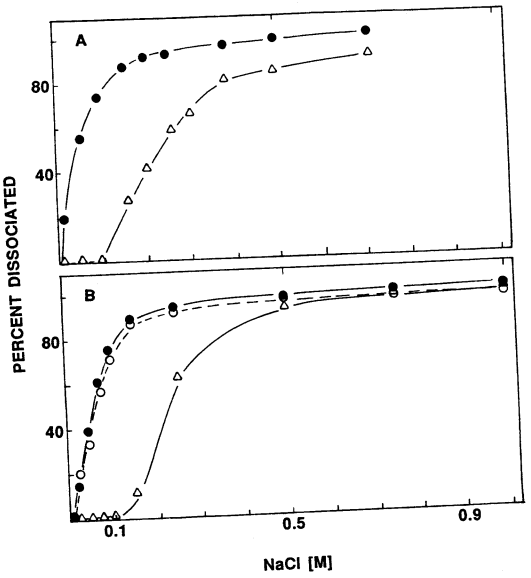

Fig. 1 The dissociation of cytochrome *c* (●) and cell wall-associated β-GlcNAcase (△) activity from purified potato tuber cell walls. A) Cytochrome *c* was artifactually bound to an aliquot of untreated purified cell walls and dissociated with NaCl. This was compared to the NaCl dissociation of endogenous cell wall β-GlcNAcase activity. B) The dissociation of cytochrome *c* and cell wall-associated β-GlcNAcase after rebinding to purified cell walls. Similar to Figure 1A except the purified cell walls were extracted with 3 M NaCl and washed six times to remove the excess salt. Cyt *c* (●), cytosolic β-GlcNAcase (○), and cell wall β-GlcNAcase (△) were bound to the extracted cell walls and then dissociated with increasing NaCl concentrations.

that used to release the equivalent amount of cyt *c*. Because these experiments were performed on untreated purified cell walls, it could be argued that the cyt *c* could not bind to sites already occupied by the cell wall enzymes and hence the dissociation curves would not be similar in this case. To rule out this possibility, cell walls treated with 3 M NaCl were washed six times with cold deionized-distilled water. One third of the sample was used to bind cyt *c*, another third was used to rebind the dialyzed salt-extracted cell wall β-GlcNAcase and the last portion was used to bind the cytosolic form of β-GlcNAcase. However, to get the cytosolic enzyme to bind to the cell wall, the binding solution was buffered at pH 4.0 with 25 mM sodium citrate-citric acid. The cytosolic enzyme and cyt *c* both dissociated at lower concentrations of NaCl (Fig. 1B) when compared to the rebound cell wall enzyme. All three proteins had the same opportunity to bind to any available site and yet the non cell wall proteins did not bind as tightly as the endogenous cell wall enzyme.

Effects of various salts on the dissociation of cytochrome c and β-GlcNAcase from cell walls—Cytochrome *c* was bound to purified cell walls and then dissociated with increasing ionic strength using monovalent, divalent, and trivalent cations (Fig. 2). At low ionic strength, the trivalent cation was more efficient than the divalent cation and the divalent cation was more efficient than the monovalent cation when comparing their dissociation curves (Fig. 2). Ammonium sulfate was also used to dissociate cyt *c* and at low ionic strength, it was very inefficient at solubilizing the protein. At high ionic strength (I=1.5) all salts were equally efficient at dissociating artificially bound cytochrome *c* (data not shown).

Similar effects were observed when the cell wall β-GlcNAcase was dissociated with LiCl, $CaCl_2$ and $LaCl_3$. At low ionic strength, the solubilization by La>Ca>Li (Fig. 3) however, at higher ionic strengths, we observed less activity with divalent and trivalent cations. The lower activity at higher ionic strength was due to loss of enzyme activity instead of less efficient solubilization. This was determined by directly assaying the treated walls for enzyme activity. Table 1 shows that if the cell wall enzyme was extracted when I=1.5, the monovalent salts and ammonium sulfate were most effective at dissociating an active β-GlcNAcase.

The role of pH in the dissociation of cell wall β-GlcNAcase—The role of pH in the dissociation of the cell wall enzyme was studied by using a potassium phosphate buffer between pH 4.5 to 8. The dissociation was performed between pH 4.5 to 8 at 50 mM, 250 mM, 500 mM, and 1,000 mM (Fig. 4). The actual pH readings recorded in Figure 4 were determined directly by placing the electrode into the stirred cell wall buffer suspension. At 50 mM buffer, very little dissociation of the enzyme occurred at or below pH 6.5. As the ionic strength or molarity of the buffer was increased to 0.5 M, considerably more enzyme was released at acidic pH. At 1 M phosphate buffer, the amount of enzyme released was similar throughout the pH range tested.

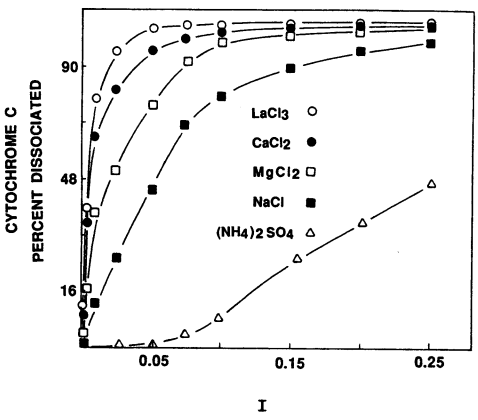

Fig. 2 Cytochrome *c* was artifactually bound to purified potato tuber cell walls and then dissociated with increasing concentrations of monovalent, divalent, and trivalent salts. I=ionic strength.

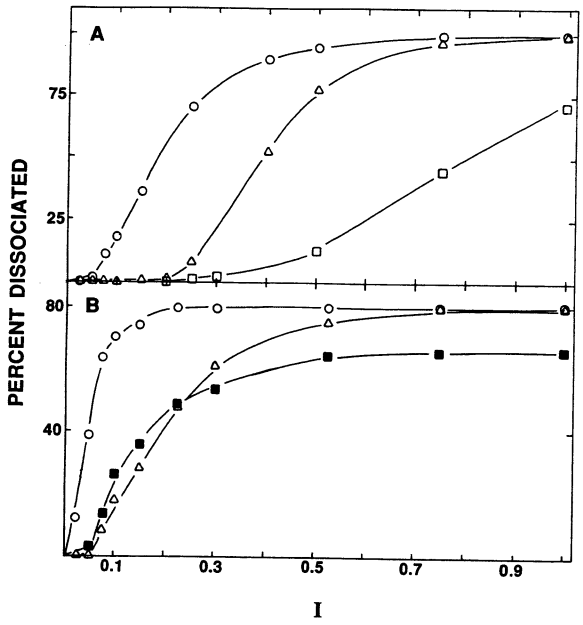

Fig. 3 The dissociation of cell wall-associated β-GlcNAcase activity from purified, extracted potato tuber cell walls with various salts. The dissociation of the endogenous cell wall enzyme was directly compared to the artifactually bound cytosolic β-GlcNAcase activity. A) Cytosolic β-GlcNAcase (○ LiCl); Cell wall β-GlcNAcase (△ LiCl; □ $(NH_4)_2SO_4$). B) Cytosolic β-GlcNAcase (○ $CaCl_2$); Cell wall β-GlcNAcase (△ $CaCl_2$; ■ $LaCl_3$). I=Ionic strength.

Table 1 The extraction of potato tuber cell wall-associated β-*N*-acetylglucosaminidase activity with various salts at the same ionic strength

Salt	μmoles fraction^{-1} h^{-1}
1.5 M LiCl	14.26 (±0.14)
1.5 M KCl	12.26 (±0.14)
1.5 M NaCl	12.89 (±0.35)
0.5 M $(NH_4)_2SO_4$	13.45 (±0.23)
0.5 M $CaCl_2$	9.46 (±0.22)
0.5 M $MgCl_2$	9.20 (±0.31)
0.25 M $LaCl_3$	5.74 (±0.38)

Ten mls of cell wall suspension were stirred at room temperature for 1 h. Aliquots of the supernatants were assayed for enzyme activity and the total activity released was calculated. Standard error in parentheses.

Binding of proteins with various isoelectric pH—Binding studies were performed with lysozyme (pI=11.0, mol wt=14 kDa, α-lactalbumin (pI=4.5, mol wt=14 kDa), β-lactoglobulin (pI=4.9, mol wt=36 kDa) and bovine serum albumin (pI=5.3, mol wt=66 kDa). Of these proteins, only lysozyme (data not shown) would bind to the wall in an unbuffered suspension.

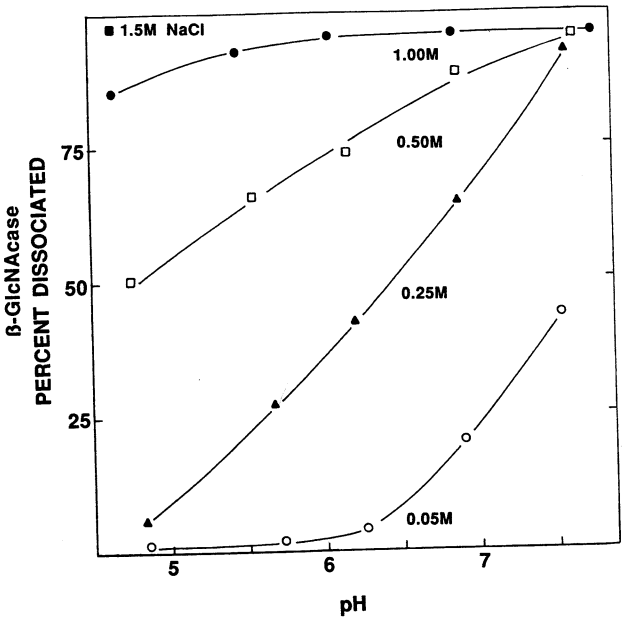

Fig. 4 The effects of pH and ionic strength on the dissociation of potato tuber cell wall-associated β-GlcNAcase activity. The dissociations were performed at various pH with a potassium phosphate (monobasic and dibasic) buffer system with increasing molarity. The pH readings were determined by placing the electrode in the buffered cell wall suspensions.

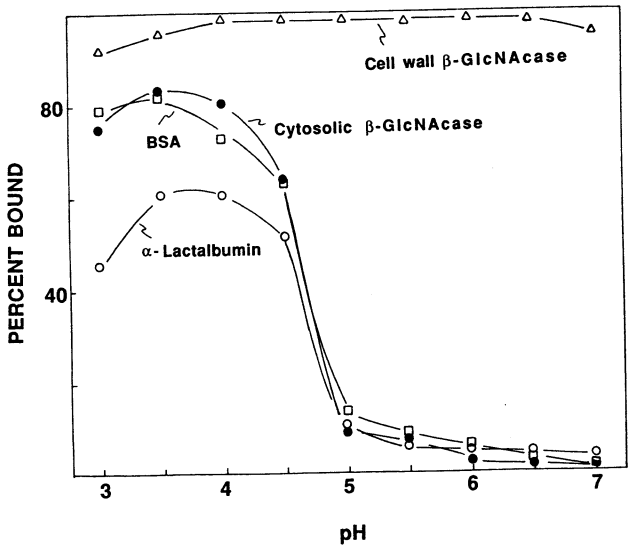

Fig. 5 The binding of acidic proteins at various pH to purified extracted potato tuber cell walls. The binding of BSA, α-lactalbumin, and cytosolic β-GlcNAcase were used as acidic proteins and compared to the basic cell wall β-GlcNAcase activity. A sodium citrate-citric acid buffer system (25 mM) was used for this study.

It has been reported that acidic proteins can bind to cell walls (Masuda and Sugawara 1978, Barcelo et al. 1988), so binding studies were performed at various pH with potato cell walls. BSA, α-lactalbumin, and cytosolic β-GlcNAcase were the acidic proteins used and were compared to the binding of the basic cell wall β-GlcNAcase. Between pH 3 to 4.5, 60 to 80% of the total BSA or α-lactalbumin added bound to the wall. At pH 6 or greater, little or no binding occurred (Fig. 5). Similarly, the cytosolic β-GlcNAcase (pI=5.2) would bind under acid conditions but would not bind above pH 6. BSA, α-lactalbumin and cytosolic β-GlcNAcase at acidic pH became protonated below their pI values and were able to bind to the negative charge of uronic acid residues of the cell wall. In contrast, the rebinding of the cell wall β-GlcNAcase was not pH dependent (Fig. 5) when the binding was performed at room temperature or at 4°C (data not shown). If binding experiments were performed at 38°C, there was an apparent loss of binding at pH 3 and pH 7 to 8. This turned out to be not an actual loss of binding but a loss of enzyme activity due to the presence of a protease or proteases in the original cell wall extract. We are currently trying to purify and characterize this cell wall associated proteolytic activity.

Discussion

It has been reported that appropriate pH as well as

ionic strength are both necessary to dissociate proteins from cell walls (Masuda and Sugawara 1978, Nakagawa et al. 1971). By performing salt dissociation curves with increasing concentrations of a potassium phosphate buffer system, the effect of pH as well as ionic strength could be evaluated. At low ionic strength buffer, about 45% of the total β-GlcNAcase activity could be dissociated at pH 7.5 to 8 (Fig. 4) but little or no enzyme was released at or below pH 6.5. At a buffer concentration of 250 mM, almost 95% of the enzyme could be dissociated at pH 7.5 to 8 and enzyme was beginning to be released at lower pH. With 1 M buffer, ionic strength appeared to override the effect of pH. This experiment indicated that ionic strength was more important than pH for dissociating cell wall bound enzymes as long as the molarity of the buffering system (1 M or greater with a potassium phosphate buffer) was high. If a 0.5 M potassium phosphate buffer is used to extract cell wall proteins, an alkaline pH is necessary to insure maximum dissociation. The results in Figure 4 clearly indicate that cell wall proteins can be effectively dissociated from plant cell walls without buffer in a high salt solution.

To determine if salt specificity played a role in the dissociation of cell wall enzymes, salt dissociation curves were performed with mono-, di-, and trivalent cations. The salt dissociation curves for cyt *c* clearly showed a shift to the left (at low ionic strength) as the charge on the cation increased from +1 to +3. As the ionic strength was increased to 1.5 (data not shown), all of the salts tested were equally effective in dissociating this protein.

When these experiments were repeated on the cell wall associated β-GlcNAcase, similar results were obtained at low ionic strength. The dissociation was greater with the higher charge on the cation. However, at high ionic strength, divalent and trivalent cations inhibited the cell wall associated enzyme (Fig. 3). For future studies with cell wall enzymes, it would appear that extraction with monovalent salts with ionic strength greater than 1 would be the most efficient way to extract active cell wall enzymes.

Salt dissociation studies showed two other interesting observations. There was a distinct displacement of the dissociation curve of the endogenous enzyme compared to the exogenously or artifactually bound proteins. Regardless of the type of salt used in the dissociation assay, the cytosolic β-GlcNAcase was always removed at lower salt concentration than the endogenous β-GlcNAcase (compare Figs. 2 and 3). These results confirm the earlier report by Masuda and Sugawara (1978) where they compared the NaCl dissociation of cell wall invertase to the artifactually bound cytosolic invertase. The basic isozymes of the cell wall are more tightly bound than the acidic cytosolic isozymes and can be readily distinguished from each other by salt dissociation experiments. Our results further showed that an artifactually bound basic protein (cyt *c*) does not bind as tightly as endogenous basic cell wall proteins.

Secondly, ammonium sulfate was the least efficient salt used to dissociate endogenous or artificially bound proteins at low ionic strength (Fig. 2). However, at higher ionic strength (I=1.5), this salt was as effective as the monovalent cation salts (Table 1). Ammonium sulfate at this concentration (0.5 M) is approximately 15% saturation. If ammonium sulfate (0.5 to 1.0 M) is used to solubilize cell wall proteins, the filtered supernatant could then be further fractionated by adding increasing amounts of the salt. By 80% saturation, all of the cell wall extracted proteins precipitated out of solution.

Finally, it should be mentioned that the binding of proteins to purified cell walls is dependent on pH only if the protein or enzyme has an acid pI. Acidic proteins will only bind if the binding medium is at a pH near or below the pI which effectively gives these proteins a positive charge via protonation. The binding of basic proteins to the wall occurs readily in water and is not effected by changes in pH throughout the physiological range (Fig. 5). If cell walls are isolated at pH 7.5 to 8.0, at least the cytoplasmic contamination by acidic proteins can be minimized.

References

Barcelo, A.R., Pedreno, M.A., Munoz, R. and Sabater, F. (1988) Lupin peroxidases. II. Binding of acidic isoperoxidases to cell walls. *Physiol. Plant.* 73: 238–244.

Huber, D.J. and Nevins, D.J. (1982) Exoglucanases from *Zea mays* L. seedlings: Their role in β-D-glucan hydrolysis and their potential role in extension growth. *Planta* 155: 467–472.

Jansen, E.F., Jang, R. and Bonner, J. (1960) Binding of enzymes to Avena coleoptile cell walls. *Plant Physiol.* 35: 567–574.

Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87: 206–210.

Masuda, H., Komiyama, S. and Sugawara, S. (1988) Extraction of enzymes from cell walls of sugar beet cells grown in suspension culture. *Plant Cell Physiol.* 29: 623–627.

Masuda, H. and Sugawara, S. (1978) Adsorption of cytoplasmic and wall-bound saccharase of sugar beet seedlings to cell wall. *Agric. Biol. Chem.* 42: 1479–1483.

Nagahashi, G. and Garzarella, L. (1988) An extrinsic marker for higher plant cell walls. *Protoplasma* 143: 165–169.

Nagahashi, G. and Seibles, T.S. (1986) Purification of plant cell walls: Isoelectric focusing of calcium chloride extracted enzymes. *Protoplasma* 134:102–110.

Nagahashi, G., Tu, S-I., Fleet, G. and Namgoong, S.K. (1990) Inhibition of cell wall-associated enzymes in vitro and in vivo with sugar analogs. *Plant Physiol.* 92: 413–418.

Nakagawa, H., Sekiguchi, K., Ogura, N. and Takehana, H. (1971) Binding of tomato pectinesterase and β-fructofuranosidase to tomato cell wall. *Agric. Biol. Chem.* 35: 301–307.

Scott, T.K. and O'Neill, R.A. (1984) Cell wall proteins extracted from suspension-cultured cells. *In* Structure, Function and Biosynthesis of Plant Cells Walls. Edited by Dugger, W.M. and Bartnicki-Garcia, S. pp. 268–283. Waverly Press, Baltimore, MD.

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